KINETICS OF PRENEOPLASTIC EPITHELIA OF THE INTESTINAL MUCOSA INDUCED IN BUFFALO RATS BY ORAL ADMINISTRATION OF N,N'-2,7-FLUORENYLENEBISACETAMIDE

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A diet containing 0.025% of N,N'-2,7-fluorenylenebisacetamide (2,7-FAA) was administered orally to 18 Buffalo rats for 3 months and to 21 rats for 5 months which corresponded to non-neoplastic stage and preneoplastic stage of carcinogenesis of the intestinal mucosa, respectively. The mitotic index, labeling index, and generation time of the epithelia of the intestinal mucosa of these stages were examined by autoradiography.

1) The mitotic index and labeling index decreased in the rats fed 2,7-FAA for 3 months than those of the non-treated controls.
2) Decrease of both indices was also observed in the rats fed 2,7-FAA for 5 months, except for the proximal part of the colon.
3) Around 1-hr prolongation of the generation time was observed in the epithelia of rats fed 2,7-FAA for 3 months compared with the non-treated control rats, and this was mainly due to the prolongation of the duration of the G1 phase.
4) Two- or three-hour prolongation of the generation time was observed in the epithelia of rats fed 2,7-FAA for 5 months compared with the non-treated control rats, and this was mainly due to the prolongation of the duration of the S phase.

An aromatic amine compound, N,N'-2,7-fluorenylenebisacetamide (2,7-FAA), has an ability to induce neoplasm in several organs, such as the liver, mammary gland, lung, intestine, bone marrow, glandular stomach, etc., when it is administered orally or intraperitoneally to Buffalo or A × C rats.9) Previously we reported that the intestinal tumors developed in 50% of Buffalo rats by this carcinogen. No tumors were observed in the rats fed 2,7-FAA for 3 months and initial tumor appeared at over 5 months.17) Although the frequency is somewhat lower than that by dimethylhydrazine,21 cycasine,5) or methylazoxymethanol,11) oral administration of 2,7-FAA in the form of a diet is a more simple and easy method than other methods, and the multiple tumors developing in the small intestine and colon were suitable for the study of cell kinetics. Thus this method offers a good tool for the study on tumorigenesis of the intestine.

In the present work, the mitotic index, labeling index, and generation time of the intestinal epithelia in the preneoplastic stages induced by 2,7-FAA were analyzed by autoradiography.

MATERIALS AND METHODS

Animals A total of 84 female Buffalo rats were obtained from the Hoshino Animal Farm, Saitama, and were used from 7 weeks of age. Eighteen rats were fed a diet CE-2 (CLEA Japan Inc., Tokyo) containing 0.025% 2,7-FAA (Tokyo Kasei Co.) continuously for 3 months and 18 control rats of comparable age were fed CE-2 diet only. Both groups of rats were killed under ether anesthesia.
at various time intervals from 0.5 to 22 hr after intraperitoneal injection of 1 μCi/g body weight of \(^3\)H-thymidine (13.0 mCi/mmol; Daiichi Pure Chemicals Co., Tokyo). The 21 rats fed 2,7-FAA for 5 months and 27 control rats were sacrificed at various time intervals from 0.5 to 42 hr after the \(^3\)H-thymidine injection.

The whole intestine from the duodenum to the rectum was removed immediately after the animals were killed. It was stretched on a wooden board for measuring the length and then the lumen was opened with scissors. Based on results of the previous study, the intestine was devided into 10 sections.\textsuperscript{17} Sections 1 and 2 correspond to the duodenum, Section 3 to the jejunum, Sections 4, 5, and 6 to the ileum, Section 7 to the cecum, Section 8 to the proximal part of the colon, Section 9 to the middle part of the colon, and Section 10 to the distal part of the colon and the rectum. From each section, the intestines were cut out into pieces of about 2.0 × 0.3 cm in size along the major axis. The serosal side of the pieces was placed on a filter paper for adhesion to prevent coiling up of the specimens and then fixed in 10% Formalin solution.

**Preparations for Autoradiography** One week after the fixation in Formalin, the cut tissues were embedded in paraffin blocks by a routine procedure. Sections of 3-μm size were coated with nuclear track emulsion (Kodak NTB-2) by the dipping method. After 1 week of exposure, the specimens were developed with Kodak D-19 for 5 min at 18°, development stopped with 3% \(K_2Cr_2(SO_4)_3\cdot 24H_2O\) for 2 min, fixed in acidic fixative for 15 min, and then washed with tap water for 30 min. The specimens were then stained with Hematoxylin and Eosin.

**Method of Calculations** Specimens prepared for autoradiography were used for the calculation of mitotic index, labeling index, and generation time of epithelial cells of intestinal mucosa. Methods used for the calculations were as follows:

- **Mitotic Index:** More than 5,000 epithelial cells were counted from top of the villus to the neighboring villi. Percentage of mitotic cells among the whole epithelial cells was calculated from its results.
- **Labeling Index:** More than 1,000 epithelial cells were counted from top of the villus to the neighboring villi for estimating the percentage of labeled cells. The labeled cell was defined as the cell in which more than 5 silver grains were visible on the nucleus.
- **Generation Time:** Generation time of the intestinal epithelia was calculated by the labeled mitosis curve method reported by Quastler and Sherman.\textsuperscript{14} Namely, 200 mitotic epithelial cells were counted in each autoradiographic specimen from 0.5 to 42 hr after the intraperitoneal injection of \(^3\)H-thymidine and the percentage of labeled mitotic cells among them was estimated. The durations of G1, S, G2, and M phases were calculated from these labeled mitosis curves.

**RESULTS**

**Mitotic Index** In almost every section of the intestine, a decrease in the mitotic index was observed in rats fed 2,7-FAA for 3 and 5 months compared to that of the non-treated control group. In the 3-month group, the decrease was more marked in the ileum (Sections 4, 5, and 6) than in other sections of the intestine. No significant difference, however, was noticed in the duodenum.

In the 5-month group, average values of the mitotic index were also lower in the 2,7-FAA fed rats than in the non-treated controls, except in the proximal part of the colon (Section 8), which was the most susceptible part for the development of tumors from our previous work.\textsuperscript{17} With the exception of the cecum (Section 7) the mitotic index was lower in the 5-month group than in the 3-month group (Fig. 1).

**Labeling Index** In the 3-month group, a more marked decrease in the labeling index than the mitotic index was observed in every section of the intestine, with the exception of proximal part of the colon (Section 8). In general, similar decrease in the labeling index compared with that of the non-treated control rats was observed in the rats of the 5-month group, but such a decreasing tendency was not recognized in the duodenum (Sections 1 and 2), cecum (Section 7), and proximal part of the colon (Section 8) (Fig. 2).

**Generation Time** In every section of the intestine, curves of the fractions of labeled mitosis reached their peak at about 2 hr after the injection of \(^3\)H-thymidine. The high level of the curves continued for about 5 hr and then began to decline, both in the 2,7-FAA fed rats and in the non-treated control rats. The maximum level of the peak, however,
Fig. 1. Mitotic index of epithelial cells in each section of the intestinal mucosa from rats treated with 2,7-FAA for 3 or 5 months
- FAA group  ○ Control group
Vertical bar indicates standard error.

Fig. 2. Labeling index of epithelial cells in each section of the intestinal mucosa from rats treated with 2,7-FAA for 3 or 5 months
- FAA group  ○ Control group
Vertical bar indicates standard error.

Fig. 3. Generation time of epithelial cells in each section of the intestinal mucosa from rats treated with 2,7-FAA for 3 months
-------- Control  ---- 2,7-FAA
Table I. Generation Time of Epithelial Cells in Each Section of the Intestinal Mucosa from Rats Treated with 2,7-FAA for 3 Months

<table>
<thead>
<tr>
<th>Section of the intestine</th>
<th>G1+0.5M FAA</th>
<th>G1+0.5M Control</th>
<th>S FAA</th>
<th>S Control</th>
<th>Generation time (hr) FAA</th>
<th>Generation time (hr) Control</th>
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<td>11.8</td>
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<tr>
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<td>6.2</td>
<td>6.6</td>
<td>12.2</td>
<td>11.0</td>
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<td>3.6</td>
<td>6.4</td>
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<td>11.6</td>
<td>12.0</td>
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<td>5</td>
<td>5.0</td>
<td>5.0</td>
<td>6.2</td>
<td>6.6</td>
<td>12.8</td>
<td>13.2</td>
</tr>
<tr>
<td>6</td>
<td>3.6</td>
<td>4.6</td>
<td>7.4</td>
<td>7.4</td>
<td>12.6</td>
<td>13.4</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate the average time

Table II. Generation Time of Epithelial Cells in Each Section of the Intestinal Mucosa from Rats Treated with 2,7-FAA for 5 Months

<table>
<thead>
<tr>
<th>Section of the intestine</th>
<th>G1+0.5M FAA</th>
<th>G1+0.5M Control</th>
<th>S FAA</th>
<th>S Control</th>
<th>Generation time (hr) FAA</th>
<th>Generation time (hr) Control</th>
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</thead>
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<tr>
<td>Small intestine</td>
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<td>4.6</td>
<td>7.4</td>
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<tr>
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<td>4.0</td>
<td>8.0</td>
<td>6.6</td>
<td>13.4</td>
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<tr>
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<td>6.5</td>
<td>14.4</td>
<td>12.8</td>
</tr>
</tbody>
</table>

In the 5-month group, more marked prolongation of the generation time was observed than in the 3-month group, and it became apparent that the delay was due not only to the prolongation of the duration of G1 phase but also to that of the S phase (Fig. 4, Table II).

was slightly lower in the 2,7-FAA fed groups than in the non-treated controls.

In the small intestine of the non-treated control rats, minimum values of the curves appeared at about 10 hr after the injection, while 1 or 2 hr of delay was observed in every section of the 2,7-FAA fed groups, and it was more marked in the colon (Sections 8~10) than in the small intestine (Sections 1~6). In the colon, the delay was mainly due to the prolongation of the duration of G1 phase. Thus, start of the second wave was delayed in the 2,7-FAA fed rats (Fig. 3, Table I).
Fig. 4. Generation time of epithelial cells in each section of the intestinal mucosa from rats treated with 2,7-FAA for 5 months

- - - Control  --- 2,7-FAA
DISCUSSION

There are many reports on kinetics of the epithelial cells of normal intestinal mucosa by the use of autoradiographic techniques. Lipkin\(^7\) and Lesher\(^6\) reported that the average generation time is 11.5 hr in the epithelia of small intestine and 16.0 hr in those of large intestine in mice. Thrasher\(^16\) and Lesher\(^6\) reported that the generation time of the intestinal epithelia of the mouse was prolonged with the advance of age.

We also calculated the generation time of intestinal epithelia of the non-treated control rats at different ages comparing with the rats fed 2,7-FAA for 3 or 5 months. The generation time was 12.2 hr in the small intestine, 21.8 hr in the large intestine of the 3-month control rats, and 11.6 hr in the small intestine and 21.7 hr in the large intestine on the 5-month control rats.

It is well known that the generation time is influenced by the biological and/or environmental conditions of the cells. Prolonged generation time of Ehrlich ascites tumor cells with lapse of time after the transplantation was mentioned by Tannock.\(^15\) Hyodo-Tagoguchi\(^4\) and Garcia\(^3\) reported prolongation of the duration of S and G1 phases of the intestinal epithelia in goldfish when they were maintained at a lower temperature. Okumura and Matsuzawa\(^12\) reported that the two-fold prolongation of the generation time of intestinal epithelia was observed in germ-free CFW mice compared to the conventional mice.

In the present experiment, slight prolongation of the G1 phase was observed in the rats fed 2,7-FAA for 3 months. Labeling index and mitotic index also decreased in this group. Previously we reported the suppressed growth of the rats fed 2,7-FAA for several weeks.\(^10\) Considering these data, the elongation of G1 phase in the 3-month group seemed to be due to toxic effect on the intestinal mucosa by 2,7-FAA. In the rats fed 2,7-FAA for 5 months, each phase of the cell cycle was slightly prolonged but the prolongation was most prominent in the S phase. Pozharisski\(^13\) reported that the prolongation occurred in the S phase in the intestinal tumor, induced by 1,2-dimethylhydrazine, which has a strong carcinogenicity to intestinal mucosa. Furthermore, the administration of 2,7-FAA for 5 months corresponded to the time just before appearance of the tumor.\(^17\) Thus the prolongation of the S phase in the 5-month group seemed to be intimately correlated to the initiation of neoplastic changes.

According to Cramer\(^1\) and Miller,\(^9\) orally introduced 2-FAA was absorbed from the small intestine and reached the liver through the portal vein. In the liver, 2-FAA was converted to N-hydroxylated 2-FAA, a proximate carcinogen, by liver enzymes and then it flows down with bile juice into the duodenum or enters into the general blood circulation. Esterified N-hydroxy compounds, the ultimate carcinogen, were formed in the target organs or tissues and exert their carcinogenicity on the cells by binding with protein or DNA. From these biochemical studies, it is assumed that prolongation of the S phase of the intestinal epithelia in preneoplastic stages might be the reflection of a direct action of the ultimate carcinogen on the target cells of the intestinal mucosa. Prolongation of the S phase might also give the cells more chance to be exposed to the carcinogenic hazards. In this respect, changes like a “vicious cycle” might occur in the intestinal epithelia of the rats fed 2,7-FAA for 5 months or more.

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